Extraction and Thin Layer Chromatography of Aflatoxin B_1 in Mixed Feeds

GAIL M. SHANNON, ODETTE L. SHOTWELL, & WILLIAM F. KWOLEK[1]
Agricultural Research Service, Northern Regional Research Center, Peoria, IL 61604

A method was developed for the determination of aflatoxin B_1 in commercially prepared feeds. The method incorporates methylene chloride and citric acid solution extraction, cleanup on a small silica gel column, and thin layer chromatography for quantitation. Commercial turkey starter, catfish chow, medicated pig starter, broiler finisher, rabbit chow, horse feed, rat chow, and dog chow were investigated. The feeds were spiked with naturally contaminated corn at 4 different levels of aflatoxin B_1 (16–130 μg/kg). Three assays were run on each of the 32 combinations of feed and levels of aflatoxin. Mean recoveries were 85.9–92.8% at levels of 16.5, 32.9, 65.8, and 131.6 μg/kg. The relative standard deviation per assay was 18.6%. This method is more rapid and less involved than most previously published methods for mixed feeds.

A new method was developed for the determination of aflatoxin B_1 in commercially prepared feeds because of the great diversity that can occur in mixed feeds. Presently available methods may work very well on some feeds and not at all on others. Other drawbacks must also be considered. One method (1) involves the routine use of a 2-dimensional thin layer chromatography (TLC) that is more time consuming than desired. Another (2) uses a rather complicated purification procedure. Recoveries were only 60–65% for a third method (3). The present method, a modification of the animal tissue method (4), is comparatively simple, rapid, and inexpensive, yielding mean recoveries of aflatoxin B_1 from 85.9 to 92.8%. This research included 8 feeds, each feed spiked at 4 different B_1 levels (16.5, 32.9, 65.8, and 131.6 μg/kg). Three assays were run on each of the 32 combinations of feed and level, and the B_1 level is reported.

Feed samples were spiked with a naturally contaminated grain sample because of the adverse effects of the feed ingredients on recoveries of aflatoxin B_1 in a pure solution. This does not set a precedent (2, 3). Statistically, the standard deviation for recovery values in 9 assays of the spiking grain does not differ significantly from that of the spiked feed samples.

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[1] North Central Region, ARS, stationed at the Northern Regional Research Center.

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METHOD

Reagents and Apparatus

(a) *Solvents.*—ACS grade glacial acetic acid, acetone, acetonitrile, benzene, dichloromethane, ethyl ether (0.01% ethanol and 1 ppm butylated hydroxytoluene), hexane (boiling range 68–69°C), tetrahydrofuran, and toluene.

(b) *Citric acid solution.*—20%. Dissolve 200 g ACS grade citric acid monohydrate in 1 L water.

(c) *Silica gel 60.*—Merck 7734. Activate 1 h in 105°C oven, add 1% water, and equilibrate overnight.

(d) *Sodium sulfate.*—Anhydrous, granular.

(e) *Diatomaceous earth.*—Hyflo Super-Cel.

(f) *Aflatoxin B_1 standard.*—Prepare in acetonitrile–benzene (2 + 98) to contain 0.5 μg/mL for either visual or densitometric analysis. Store standards in sealed glass ampules, in 0°F freezer until needed. After opening, store in 1 dram vials fitted with Teflon-lined screw caps, and keep in freezer when not in use.

(g) *Wrist-action shaker.*—Burrell, or equivalent.

(h) *Chromatographic columns.*—Glass (1.0 cm id × 50 cm) equipped with Luer nylon stopcock (Bio-Rad Econo-columns), or equivalent.

(i) *Filter paper.*—24 cm S & S No. 560, or equivalent, and 12.5 cm Whatman 934-AH glass microfiber filters, or equivalent.

(j) *Thin layer plates.*—Commercial prepoured plates (20 × 20 cm, Macherey and Nagel Sil G-25 HR), or equivalent.

(k) *Scanning spectrofluorodensitometer.*—Schoeffel SD-3000-3, or equivalent.

(l) *Single-pan balance.*—Sartorius Model 2250 or Mettler P 1200 N, or equivalent.

(m) *Mixer.*—Vortex Model K-500-2, or equivalent.

(n) *Concentrator.*—SMI Model No. 6610 (Scientific Manufacturing Industries, Inc., Emeryville, CA 94608), or equivalent.

Samples

Commercial feeds were obtained locally and ground, as needed, in a Waring blender. Each feed was judged aflatoxin-free after triplicate analysis for aflatoxin B_1 by the AOAC official method for corn (5). Thin layer chromatography (TLC) of these sample extracts was quite difficult because of numerous fluorescent interferences. In some cases an ether predevelopment was sufficient for cleanup, and in others 2-dimensional TLC was necessary. All feeds used were free of aflatoxin B_1. A pre-analyzed (5), naturally contaminated corn sample was then added to the clean feed by weight, according to the B_1 level desired, to obtain the 50 g necessary for a single analysis. The presence of aflatoxin B_1 in the corn was confirmed by AOAC method **26.083** (5).

Extraction

Weigh 50 g ground and blended feed into 500 mL glass-stopper Erlenmeyer flask. Add 20 g Hyflo Super-Cel. Add 10 mL citric acid solution and 200 mL dichloromethane. Shake flask as vigorously as possible on wrist-action shaker (setting between 2.5 and 3 for Burrell) for 30 min. Filter mixture through paper (S & S 560) into 300 mL Erlenmeyer flask containing 10 g Na_2SO_4, and collect 90–100 mL. Gently swirl flask, let contents settle, and refilter solution through glass microfiber filter (Whatman 934-AH) into 100 mL graduate. Collect 40 mL for column chromatography, which represents 10 g of original feed sample.

Column Chromatography

Equip column with small plug of glass wool to provide base for silica gel. Pour column half full with dichloromethane and add 2.0 g silica gel. Add 3–4 mL dichloromethane and slurry with stainless steel (or glass) rod. Drain to settle silica and rinse sides of column with dichloromethane. When silica has settled and column still has ca 3 mL dichloromethane above packing, add 2 g Na_2SO_4 to cap column. Rinse sides of column with dichloromethane. Drain to ca 1 cm above top of column packing. Transfer extract to column and drain through column by gravity, stopping before passing below column cap. Rinse column sides with ca 2.0 mL dichloromethane and drain to column cap. Wash column with 25 mL glacial acetic acid–toluene (1 + 9) (use same graduate that was used for second filtration step), 25 mL tetrahydrofuran–hexane (1 + 3), and 25 mL acetonitrile–ether–hexane (1 + 3 + 6). Discard washes. Elute aflatoxin B_1 with 60 mL acetone–dichloromethane (1 + 4) and evaporate to near dryness under vacuum. Quantitatively transfer to 1 dram vial with Teflon-lined screw cap, and evaporate to dryness under nitrogen for TLC. Avoid overheating dry extract.

Thin Layer Chromatography

Add 500 μL (0.5 mL) acetonitrile–benzene (2 + 98) to dry sample residue in vial from column chromatography above, cap vial, and mix vigorously ca 1 min, preferably on Vortex shaker. Two solvent systems may be used for TLC because some samples may be encountered that result in inadequate separations. Routine solvent system is water–acetone–chloroform (1.5 + 10 + 90). Second system merely substitutes dichloromethane for chloroform, which results in a less polar system that, consequently, leaves interferences at a lower R_f range than aflatoxin B_1. Dichloromethane substitution should not be used if aflatoxin G_1 and G_2 are present because this system changes the order of resolution to B_1, G_1, B_2, then G_2. The lower R_f toxins may be masked by interferences. Solvent proportions may be varied to accommodate changes in laboratory conditions (e.g., use less water in high humidity; use less acetone at higher temperatures).

Preliminary.—Score plate with vertical lines 1 cm apart to result in 20 individual channels. Apply 10 μL of sample in each of 2 channels, superimposing 10 μL B_1 standard on one channel to serve as an internal standard. Also spot 10 μL B_1 standard in a blank channel. One plate will accommodate 8 samples and one standard. After developing plate in appropriate solvent system, air-dry ca 1–2 min, and examine plate under longwave UV light (365 nm). Compare sample aliquot to B_1 standard for bluish fluorescent spot at same R_f range. If present, dilute sample as needed to match standard. Proceed to quantitative plate, scored as mentioned above, applying necessary amount of sample to match B_1 standard.

Visual.—Spot aliquots of standard in amounts of 6, 8, 10, and 12 μL. Spot sample aliquots similarily, judging quantities to be spotted from preliminary plate. Develop plate in appropriate solvent system. Examine plate under longwave UV light in suitable viewing cabinet. Compare chromatograms of sample aliquots with those of standards for presence of single bluish fluorescent spot with R_f and fluorescence pattern similar to those in standards. If aflatoxins are

Table 1. Aflatoxin B_1 recoveries[a] from spiked feeds

Feed	Spiking levels, μg/kg			
	16.5	32.9	65.8	131.6
Turkey starter	15.21 (93)	30.29 (92)	61.67 (94)	123.63 (94)
Catfish chow	14.41 (88)	30.7 (93)	52.45 (80)	117.87 (90)
Pig starter	13.32 (81)	27.38 (83)	56.05 (85)	134.8 (102)
Broiler finisher	17.41 (106)	32.69 (99)	70.93 (108)	112.45 (86)
Rabbit chow	14.86 (90)	30.25 (92)	52.57 (80)	118.56 (90)
Horse feed	14.97 (91)	30.15 (92)	58.39 (89)	126.18 (96)
Rat chow	15.25 (93)	26.41 (80)	56.65 (86)	117.82 (90)
Dog chow	13.72 (83)	30.6 (93)	55.22 (84)	128.93 (98)

[a] Average of 3 assays per level per feed; recovery (%) in parentheses. Least significant ratio (0.05 level) of 2 means is 1.32.

judged present in sample, match fluorescence with one of the standard spots, interpolating between standards if necessary. If aflatoxin zone in sample aliquot is more intense than 6 μL standard spot, suitably redilute sample extract, taking into account amount removed in first TLC, and repeat TLC. Calculate as follows:

$$B_1\ (\mu g/kg) = (S \times Y \times V)/(X \times W)$$

where S = μL aflatoxin standard matching unknown; Y = concentration of standard, μg/mL; V = μL of final dilution of sample extract for TLC; X = μL of sample extract matching S; W = weight of product represented by final extract for TLC (10 g).

Densitometric.—Using scored plate, spot 3 aliquots of B_1 standard and 3 aliquots of each sample (trying to match standard). Develop in appropriate solvent system. Air-dry ca 1–2 min, then scan on densitometer with settings of 365 nm for excitation and 445 nm for emission. Calculate peak areas from strip recorder chart or use in-line computer figures. Calculate as follows:

$$B_1\ (\mu g/kg) = (B \times Y \times V)/(Z \times W)$$

where B = average area of aflatoxin peaks per μL in sample aliquots; Y = concentration of aflatoxin standard, μg/mL; V = μL final volume (500 × dilution) of sample extract for TLC; Z = average area of aflatoxin peak per μL in standard aliquots; W = weight of product represented by final extract for TLC (10 g).

Results and Discussion

This study was originally attempted with a pure solution of aflatoxin B_1 for spiking. After several mixed feeds were spiked and analyzed, it was found that recovery levels were not consistent, ranging from 37 to 147%. Often, within the same feed, the range could vary by 50% or more. Feed additives evidently can have nonreproducible effects on pure aflatoxin. It was decided to redesign the experiments and use a naturally contaminated corn sample for spiking the mixed feeds. Recovery ranges from these samples were satisfactory (Table 1).

The analysis of variance for these data is shown in Table 2. Log B_1 was used because a very wide range in spiking levels (8-fold) was examined.

The analysis of Table 2 indicates the following:

(a) There is no significant variation among feeds in mean B_1 (averaged over 4 levels × 3 samples); the feed mean square of 0.00583 equals the variation among assays.

(b) There is a highly significant linear trend (L_L) with spiking level. Deviations from a linear trend are not significant (L_R).

(c) Regardless of feed, the relation between B_1 level and spiking level is linear. There was no evidence that spiking level interacted with feed.

(d) The standard deviation among assays is $\sqrt{0.00551} = 0.07423$ in log units. The relative

Table 2. Analysis of variance of log B_1 for 8 feedstuffs with 4 spiking levels of B_1

Source of variation	df[a]	Mean square
Feed (F)	7	0.00583 ns
Level (L)	(3)	
Linear L_L	1	11.05269**
Remainder L_R	2	0.00690 ns
F × L interaction	(21)	
FL_L	7	0.00390 ns
FL_R	14	0.00288 ns
Among assays	64	0.00551
	95	

[a] df = degrees of freedom.
** Significant at 0.01 level; ns = not significant.

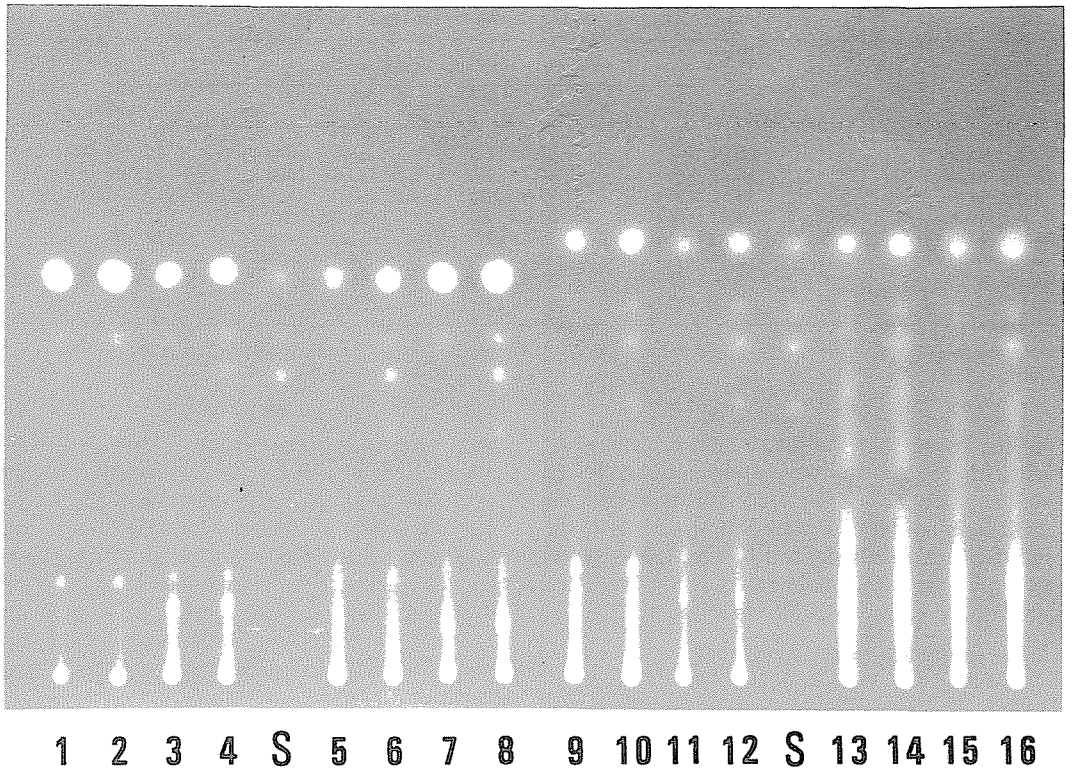

Figure 1. Composite picture of 2 TLC plates showing spiked feed extracts spotted alone and in admixture (even numbered spots) with B_1 standard as follows: 1 & 2 = turkey starter, 131.63 μg B_1/kg; 3 & 4 = broiler finisher, 65.82 μg B_1/kg; 5 & 6 = pig starter, 32.9 μg B_1/kg; 7 & 8 = horse feed, 131.63 μg B_1/kg; 9 & 10 = catfish chow, 32.9 μg B_1/kg; 11 & 12 = dog chow, 32.9 μg B_1/kg; 13 & 14 = rabbit chow, 32.9 μg B_1/kg; 15 & 16 = rat chow, 32.9 μg B_1/kg; S = aflatoxin B_1 standard, 0.5 μg/mL.

standard deviation per assay is $100\ (1-10^{0.07423}) = 18.6\%$.

In addition, there is no significant evidence that variation among assays depended on feed type, although catfish chow and pig starter had somewhat larger variances than other feeds.

The least significant ratio significantly greater than 1.00 at the 0.05 level is 1.322, assuming 3 assays. This value applies to the sample means for a feed-level combination.

The mean B_1 recoveries for all mixed feeds are shown in Table 3. The computed overall relation between spiking level (X) and B_1 was

$$B_1 = 0.86676X^{1.0083}$$

The exponent 1.0083 is not significantly different from unity. If an assumed exponent of unity is used, the model becomes

$$B_1 = 0.8947X$$

Thus, recovery is estimated at 89.5%. In an unpublished study, our mixed feeds method and Romer's mixed feeds method (2) were used to analyze 108 chicken ration samples, 27 of which were positive. The arithmetic coefficient of variation for all positive samples analyzed by the Romer method was 33.3% and by our method 20.9%. The limit of detection of aflatoxin B_1 is less than 2 μg/kg in most feeds with the NRRC method. This was accomplished by spiking blank feed extracts with standard solution and running on a TLC plate.

Figure 1 pictures the various feed extracts on

Table 3. Mean B_1 recovered at 4 spiking levels

Spiking levels, μg/kg	Geometric mean B_1 recovered, μg/kg	Rec., %
16.45	14.78	89.8
32.9	29.44	89.5
65.8	56.54	85.9
131.6	122.18	92.8
Maximum level significantly different from 100		93.3

a TLC plate. Detection of aflatoxins B_1 and B_2 is no problem, but occasionally pig starter has a blue fluorescent zone very near B_1. This can be eliminated by replacing chloroform with dichloromethane in the TLC developing solvent. Then the interfering substance moves ahead of B_1. Determination of G_1 and G_2 would be very difficult due to low R_f materials.

The use of glass fiber filters for the last filtration step is very important. This removes column-clogging fines and permits a fast flow rate that lessens evaporation problems.

Preliminary experimentation with high pressure liquid chromatography (HPLC) was tried on some of the sample extracts, but the results were not satisfactory. We employed a water adduct derivatization with trifluoroacetic acid before injection, and much of the lower R_f material (by TLC) caused interferences by HPLC. This area warrants more research.

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